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EXPERIMENTAL STUDY

Pituitary transcriptome profile of liver cancer mice with different syndromes reveals the relevance of pituitary to the cancer and syndromes

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Abstract

OBJECTIVE: To investigate the relevance of the pituitary to liver syndromes and cancer by studying the pituitary transcriptome profile in liver cancer mice with different syndromes.

METHODS: The quantitative four diagnosis and syndrome differentiation methods were used to screen normal control mice without syndromes (NC), liver cancer mice with poisonous pathogenic factors syndrome (PPFS), and *Qi* deficiency syndrome mice (QDS). An Affymetrix GeneChip Mouse Exon 1.0 ST Array was performed to detect the gene expression of different groups. Gene clustering was applied to analyze the gene expression patterns of the PPFS and QDS groups compared with the NC group. The transcriptional networks analysis tool, FunNet, was used to enrich the biological categories of differentially expressed genes in the PPFS and QDS groups.

RESULTS: Biological categories of differentially expressed genes showed that excessive metabolism and extracellular matrix interaction, insufficient communication of cells (especially nerve cells), and the bidirectional regulation of genetic information processing were enriched in both syndromes. However, the degree of excessive metabolism in the PPFS group was higher than that in the QDS group. The hyperfunction of cancer and infection, and the hypofunction of the nervous and endocrine systems were obvious in the QDS group.

CONCLUSION: The pituitary plays an important role in the development of liver cancer and syndromes. This study further studied the role of the pituitary in the combination of disease and syndromes.

Key words: Liver neoplasms; Syndrome; Pituitary gland; Transcriptome

INTRODUCTION

Major advances have been made in the management of primary hepatic carcinoma (hereafter referred to as liv-

er cancer) in recent decades. Nevertheless, liver cancer remains the second most common cause of death from cancer worldwide.[1] More than fifty percent of liver cancer patients worldwide live in China, which is a significant public health burden. Five therapeutic methods including resection, transplantation, radiofrequency ablation, chemoembolization, and sorafenib are used to treat liver cancer. In addition, some personalized medicine approaches are used to complement the shortfalls of these methods.[2] Traditional Chinese Medicine (TCM) is a personalized medicine system based on the theory of syndrome differentiation and treatment, which plays an important role in the treatment of most diseases including liver cancer. The foundation of syndromes and the mechanisms of Chinese herbs in liver cancer treatment are still unclear, but their curative effects have been identified.[3,4]

Experiments indicate that both syndrome, especially deficiency syndrome, and cancer are closely related to the neuro-endocrine-immune network (NEIN).[5,6] However, there are not many reports on the relationship between NEIN and liver cancer. We have previously studied this relationship.[7,8] We aimed to further study the role of the pituitary, an important tissue of NEIN, in the development of liver cancer and syndromes. This was done by mining the pituitary transcriptomic features of liver cancer mice with different syndromes.

MATERIALS AND METHODS

Animals and modeling

Two hundred and fifty healthy male Kunming (KM) mice of clean grade, weighing (25±1) g, were bought from Shanghai SLAC laboratory animal Co., Ltd., (certificate of quality No. SCXK [hu] 2008-0003) and housed at a controlled temperature (22℃±2℃) and humidity (40%-60%), with a 12-h light-dark cycle, and were allowed access to food and water ad libitum. This study was approved by the experimental animal ethics committee of Shanghai University of TCM.

Ten mice were excluded for over- or under-weight before the experiment. Then the remaining two hundred and forty mice were divided into two groups: normal control group (NC, *n*=50) and model group (*n*=190). Each mouse in the model group was injected with 0.2 mL (4×107/mL) H22 tumor cells (Shanghai Institute of Materia Medica, Shanghai, China) into the right armpit subcutaneously to form the liver cancer model. The same volume of normal saline was injected into the right armpit subcutaneously of each mouse in the NC. Forty mice in the model group were removed one week after injection for their failure to form tumors. Therefore, two hundred mice were used in the final experiment.

Syndrome differentiation and mice with different syndromes screening

In our preliminary study, we found that liver cancer mice could spontaneously develop syndromes without additional syndrome building and that the syndromes could be identified by using the quantitative four diagnosis and syndrome differentiation methods established by Fang *et al*. [9,10] On the 8th day after modeling, the indexes involved in the quantitative four dig nosis method included body weight, axillary temperature, the number of cells across and vertically in open-field testing within 35 seconds, grip, the *r* value of claws and tails, tumor size and diameter, were measured.

According to the standard of syndrome differentiation,[9, 10] the degree of poisonous pathogenic factors (PPF), *Qi*, *Xue*, *Yin*, and *Yang* was calculated based on above indexes. Eight liver cancer mice with lower grip and fewer movements in open field testing, which are always used to reflect *Qi* deficiency, were screened and named as the *Qi*-deficiency syndrome group (QDS). Eight liver cancer mice with no significant differences in other indexes aside from a larger solid tumor, which is considered a poisonous pathogenic factor in TCM, were selected and named as the poisonous pathogenic factor syndrome group (PPFS). Finally, eight mice with no syndromes were selected from the 50 mice in the NC group, which was still named as the NC group. All other mice were raised continuously for other experiments.

RNA extraction, purification, and quality control

Twenty-four mice from the NC, PPFS, and QDS groups were sacrificed and the pituitary was removed. Because the pituitary of each mouse was so small (about 1.5 mg each), eight pituitaries from each group were pooled together to extract the total RNA with TRIzol (Gibco, Grand Island, NY, USA) and were purified with an RNeasy Mini Kit [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. Then, the integrity of the RNA was measured with RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA) using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All procedures were performed according to the protocols provided by the manufacturers.

Microarray detections

Three cDNA microarray detections were performed with GeneChip Mouse Exon 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) using Affymetrix GeneChip Operating System (Affymetrix, Santa Clara, CA, USA) to measure the gene expression. Shanghai Biochip Co., Ltd., (Shanghai, China) was entrusted to finish this work and provided the relative expression of 16 654 genes for analysis.

Statistical analysis

The degree of PPF, *Qi*, *Xue*, *Yin*, and *Yang* was analyzed using statistical software SPSS 15.0 (SPSS, Chica-

go, IL, USA) and expressed as mean ± standard deviation. Statistical significance was assessed using a *t*-test for the degree of PPF and one-way analysis of variance for the degree of *Qi*, *Xue*, *Yin*, and *Yang*. $P<0.05$ was regarded as a significant difference.

Differentially expressed gene screening

The expression of 16 654 genes in each chip was provided by Shanghai Biochip Co., Ltd. The differentially expressed gene screening consisted of four consecutive steps: first, the average expressed value of all genes in all groups was calculated. Second, the genes whose expression in any group was greater than the calculated average value were screened out. Third, the ratios of PPFS to NC, and QDS to NC were computed. Fourth, the criteria of differential expression were set as a ratio of differentially expressed genes was greater than 1.5 or less than 0.67.

Gene clustering representing gene expression patterns

To elucidate the gene expression patterns in both the PPFS and QDS groups, gene clustering analysis was performed with Gene Cluster 3.0 (Stanford University, Stanford, CA, USA). First, all ratios of PPFS to NC and QDS to NC were replaced by $\log_{1.5}$ (ratio) to adjust 1.5-fold up- and down-regulation (ratio was 0.67) as the same magnitude of change, but in an opposite direction. Some genes were up- or down-regulated if their $\log_{1.5}$ ratios were greater than 1.0 or less than - 1.0, respectively. Second, a datafile was prepared as recommended in the Cluster manual and it was loaded. Third, the median centering of genes was created to apply a hierarchical clustering algorithm to the ratios and genes using the Euclidean distance as the measure of similarity and complete linkage clustering. Each ratio in the cluster-ordered graph was identified by a graded color (pure green through black to pure red). The visual graph of the results from Cluster was performed using TreeView (Free Software Foundation, Boston, MA, USA).

Biological categories characterizing the transcriptomic feature of the pituitary

Based on the results from gene clustering, the gene expression patterns were different between two groups and some genes were differentially expressed. We aimed to classify these differentially expressed genes based on their functions and extract the relationships between these functions and the PPFS and QDS groups. Therefore, we used a transcriptional network analysis tool, FunNet (Inserm, Paris, France),[11,12] which relied on genomic annotations provided by Gene Ontology (GO)[13] and the Kyoto Encyclopedia of Genes and Genomes (KEGG).[14]

To meet the requirements of the file format presented by the online FunNet tutorial,[11] the expressive value of all differentially expressed genes in the PPFS and QDS groups were replaced by $\log_2$ (expressive value). Data were then searched for the unique EntrezGene ID for each gene by The National Center for Biotechnology Information (NCBI)'s EntrezGene system, which is currently the only gene identification system supported by FunNet to map genes to GO or KEGG biological categories. The annotation of differentially expressed genes was applied for two GO ontologies (cellular component and biological process), and for the KEGG categories in this paper.

RESULTS

Syndrome characteristics of different groups

Compared with the PPFS group, the degree of PPF was significantly lower in the QDS group. Compared with the NC group, the degrees of Xue and Yang were lower in both the PPFS and QDS group, but both did not meet the standard of Xue deficiency and Yang deficiency. However, the degree of *Qi* only in the QDS group was significantly lower than that in the NC group. These syndrome characteristics indicated that the PPFS and QDS groups had single syndromes, without secondary syndromes (Figure 1).

Gene expression patterns

In total, 6036 genes whose expressions in any group were more than the average of all genes were involved in the gene clustering. Many genes in the PPFS and QDS groups either consistently or inconsistently expressed in significant differences when compared with the NC group, according to the graded color (green lines indicate down-regulated genes and red lines indicate up-regulated genes). The consistent up- or down-regulated genes perhaps reflect the characteristics of liver cancer, and the genes with inconsistent variation in the two groups might be the characteristic genes of specific syndromes (Figure 2).

Transcriptomic features of the pituitary

In the PPFS group, 548 up-regulated and 207 down-regulated genes were involved in the functional analysis. Among these were 466 up-regulated and 160 down-regulated genes annotated with GO Biological Process, 496 up-regulated and 160 down-regulated genes annotated with GO Cellular Component, and 262 up-regulated and 48 down-regulated genes annotated with categories of KEGG. In the QDS group, 536 up-regulated and 221 down-regulated genes were involved in the functional analysis. Among these were 496 up-regulated and 168 down-regulated genes annotated with GO Biological Process, 481 up-regulated and 179 down-regulated genes annotated with GO Cellular Component, and 230 up-regulated and 55 down-regulated genes annotated with categories of KEGG.

Pituitary transcriptomic features related to liver cancer and syndromes

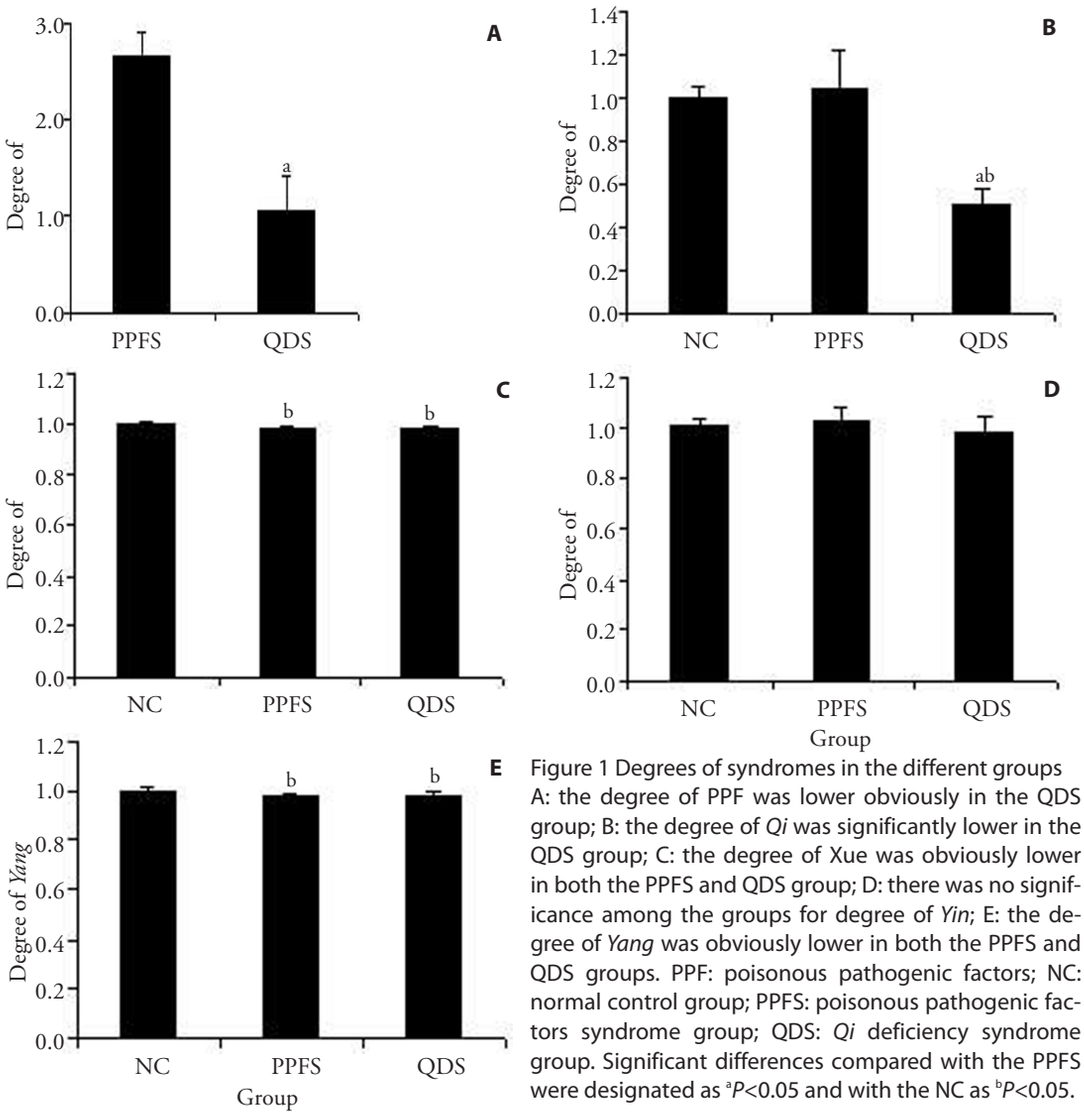

Figure 1 Degrees of syndromes in the different groups
A: the degree of PPF was lower obviously in the QDS group; B: the degree of *Qi* was significantly lower in the QDS group; C: the degree of Xue was obviously lower in both the PPFS and QDS group; D: there was no significance among the groups for degree of *Yin*; E: the degree of *Yang* was obviously lower in both the PPFS and QDS groups. PPF: poisonous pathogenic factors; NC: normal control group; PPFS: poisonous pathogenic factors syndrome group; QDS: *Qi* deficiency syndrome group. Significant differences compared with the PPFS were designated as $^{a}P<0.05$ and with the NC as $^{b}P<0.05$.

The relevant biological themes of pituitary differentially expressed genes in the PPFS and QDS groups annotated by GO Biological Process, GO Cellular Component, and KEGG are displayed in Figure 3A, B, and C and Figure 4A, B, and C.

We found that both groups had many common annotated themes. The up-regulated genes were annotated mainly by functional themes related to metabolism, especially lipid metabolism. For example: oxidation-reduction, lipid metabolic process, metabolic pathways, peroxisome proliferator-activated receptor (PPAR) signaling pathway, and fatty acid metabolism, most of these functions are always performed in the cytoplasm (cytoplasm, mitochondrion, endoplasmic reticulum, cytosol, and soluble fraction). Additionally, extracellular matrix (ECM) interactions were common [focal adhesion and ECM-receptor interaction, which generally happened in the extracellular space (extracellular space, proteinaceous extracellular matrix) and plasma membrane]. The down-regulated genes were mostly annotated by themes associated with the communication of cells, especially nerve cells. For example: cell junctions, synapses, cell projections, and the postsynaptic membrane. Additionally, the themes related to the regulation of genetic information processing were regulated bidirectionally. For example, the negative/positive regulation of transcription from RNA polymerase Ⅱ promoter was up-regulated. However, transcription, regulation of transcription, mRNA processing, RNA splicing, and response to DNA damage stimuli were down-regulated, all of which are usually processed in the cytoplasm and nucleus.

Some distinctive categories were gathered in the PPFS and QDS groups. The proportion of up-regulated genes linking metabolism (insulin signaling pathway and glycolipid, peroxisome, and pyruvate metabolisms) in the PPFS group was higher than that in the QDS group. However, the up-regulated genes related to cancer (angiogenesis, negative regulation of cell proliferation, cell proliferation, cell migration, and pathways in cancer), and infection (phagosome, Staphylococcus aureus infection, antigen processing and presentation, autoimmune thyroid disease, and viral myocarditis) were more in the QDS group. Moreover, the down-regulated genes associated with nerves (glutamatergic synapse and neurotrophin signaling pathway), and the endo-

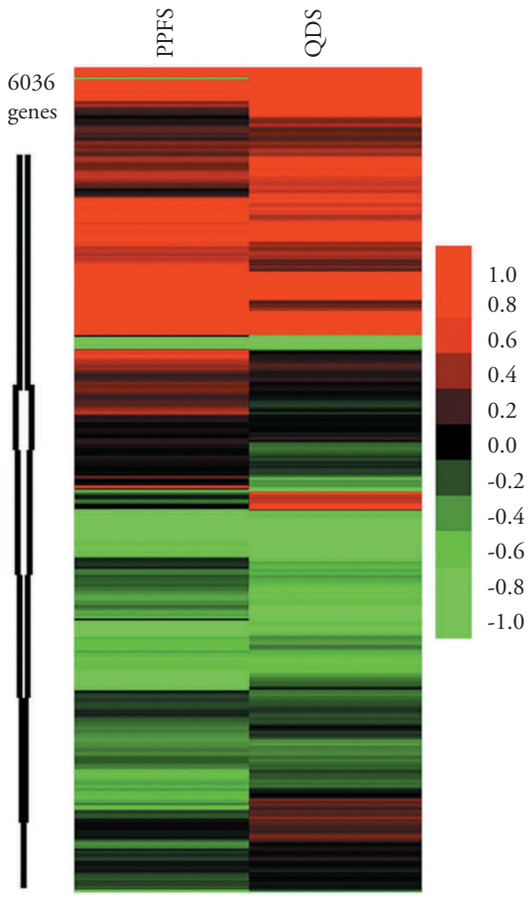

Figure 2 Gene expression patterns of the PPFS and QDS groups

A total of 6036 genes were involved in the gene clustering. Green lines represent down-regulated genes and red lines represent up-regulated genes. The lines of graded color with pure green through black to pure red (excluding pure green and pure red) indicate genes without differential expression compared with the normal control group. PPFS: poisonous pathogenic factors syndrome group; QDS: *Qi* deficiency syndrome group.

crine system (GnRH signaling pathway and gastric acid secretion) were also more in the QDS group.

DISCUSSION

Liver cancer is considered a systemic disease, which requires researchers to investigate the full pathological changes in patients. Using the combined methods of disease differentiation and syndrome differentiation in the diagnosis and treatment of liver cancer, TCM has utility in improving symptoms, reducing toxicity and side effects of chemoradiotherapy, extending lifespan, increasing quality of life, and preventing relapse and metastasis of cancer.[3]

Syndrome differentiation is the method used in TCM for disease diagnosis. For liver cancer patients, up to 30 syndromes were found in published papers reviewed by Si *et al*,[15] because no clinically consolidated standards are used to categorize syndrome names. However, all these syndromes can be divided into three categories: excess syndromes, deficiency syndromes, and deficient-excessive syndromes. In this study, two groups of liver cancer mice with common syndromes were selected: the PPFS group had excess syndrome (poisonous pathogenic factors), and the QDS group had a mixture of deficiency syndrome (*Qi* deficiency, which is the most common deficiency syndrome in the liver cancer patients[15]) and excessive syndrome (poisonous pathogenic factors). The main aim of this study was to explore the material foundation of liver cancer with these syndromes.

So far, TCM[5] and Western Medicine[6] hold that the NEIN of cancer patients is disordered in addition to the tissue with the solid tumor. One very important tissue of NEIN, the pituitary, has a close relationship with liver cancer and syndromes as Weisburger *et al* [16] and Liu *et al* [17] reported.

Liver is an important organ for maintaining the homeostasis of lipid metabolism. Most research found that the plasma lipid profiles were slightly decreased in liver cancer.[18] In the present study, we found that many up-regulated genes in the pituitary in both groups were enriched in the categories of metabolism, particularly lipid metabolism. It is speculated that the pituitary possibly increases its own lipid metabolism to compensate for the lower level of plasma lipids during liver cancer. The pituitary active extracellular matrix (ECM) interaction is generally deemed to be connected to cancer progression.[19] Additionally, liver cancer is possibly metastatic to the pituitary, though the incidence rate of this process was reported to be very low.[20] Insufficient communication of nerve cells in the pituitary likely poorly affect the ability of the neurohypophysis to store vasopressin and oxytocin from hypothalamus and its ability to release them into blood circulation under the appropriate stimuli. The regulation of genetic information processing is disordered because all abnormal processes were accompanied with changes in functional proteins translated from genetic information.

We also observed the difference between the two groups with different syndromes. Compared with the QDS group, pituitary metabolism compensation ability was stronger in the PPFS group. There were two significant differences in the QDS group from the PPFS group. First, some cancer genes were up-regulated in the QDS group, indicating that *Qi* deficiency probably facilitates tumor metastasis from liver to pituitary, which is consistent with previous reports.[21] Second, a number of infectious disease genes were affected, indicating a reduction in the immune function of the pituitary, possibly *via* nerve or endocrine. This effect is similar to the standpoint that deficiency syndromes are closely related to NEIN.[5] Both of the two specialties of the QDS group provide a theoretical foundation for the treatment of liver cancer with *Qi* deficiency by

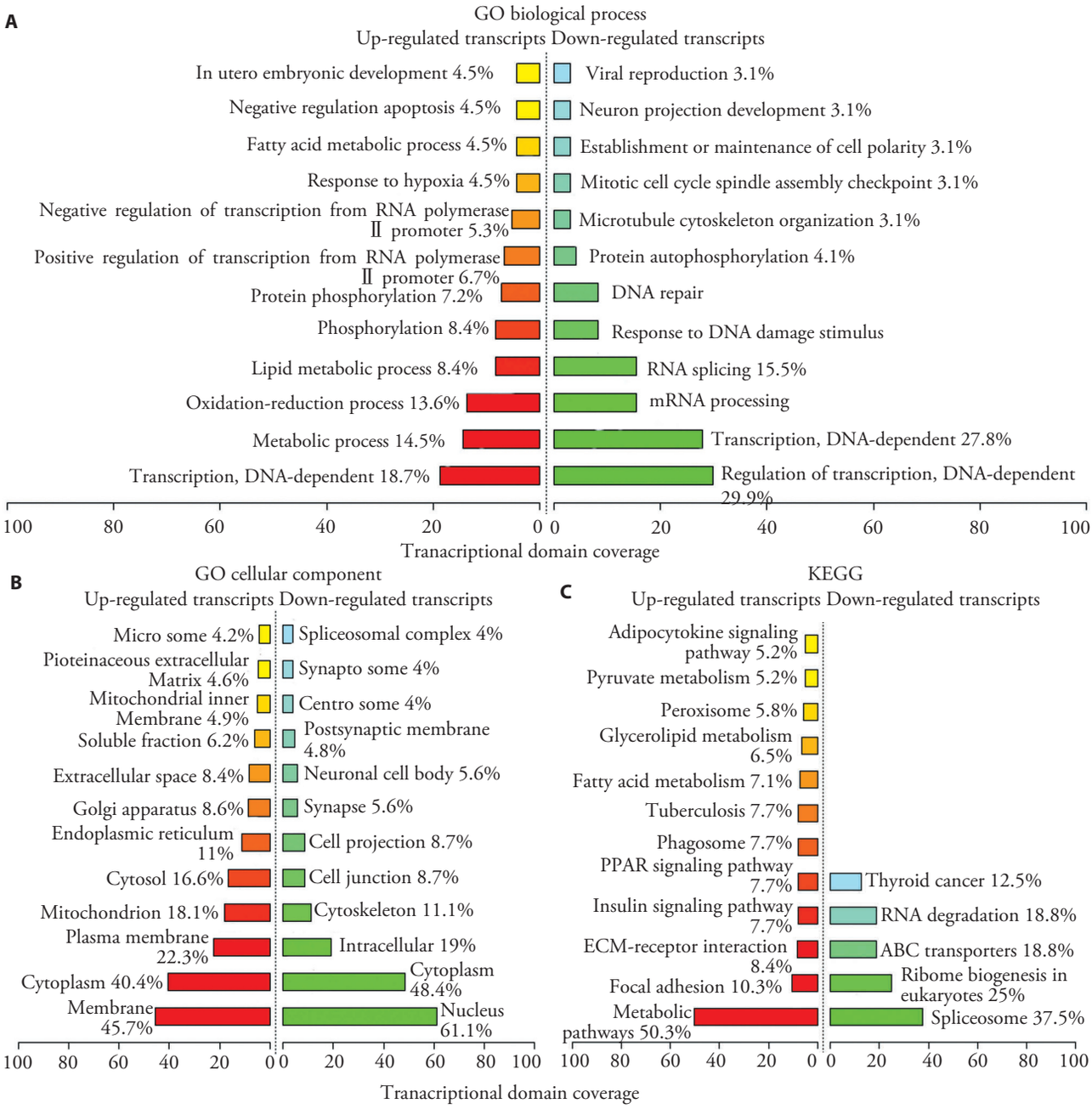

Figure 3 Biological categories of pituitary differentially expressed genes in the PPFS group

A: GO biological process annotation categories of the differentially expressed genes in the PPFS group; B: GO cellular component annotation categories of the differentially expressed genes in the PPFS group; C: KEGG annotation categories of the differentially expressed genes in the PPFS group. The percentage scale of enriched categories is presented by graded color (the categories of up-regulated genes from red to yellow versus the categories of down-regulated genes from green to blue). GO: gene ontology; KEGG: kyoto encyclopedia of genes and genomes; PPAR: peroxisome proliferator-activated receptor; ECM: extracellular matrix; ABC: ATP-binding cassette; PPFS: poisonous pathogenic factors syndrome group.

strengthening the body resistance to eliminate pathogenic factors.

Our findings of the transcriptomic features characterizing the pituitary role in liver cancer with different syndromes confirm the strong relationship between the pituitary and liver cancer and syndromes. The interaction and regulated mechanisms of these differentially expressed genes remain to be further studied. In short, our study reveals the relevance of the pituitary to disease and syndromes and opens new perspectives in understanding the role of the pituitary in the substantial foundation of combined diseases and syndromes.

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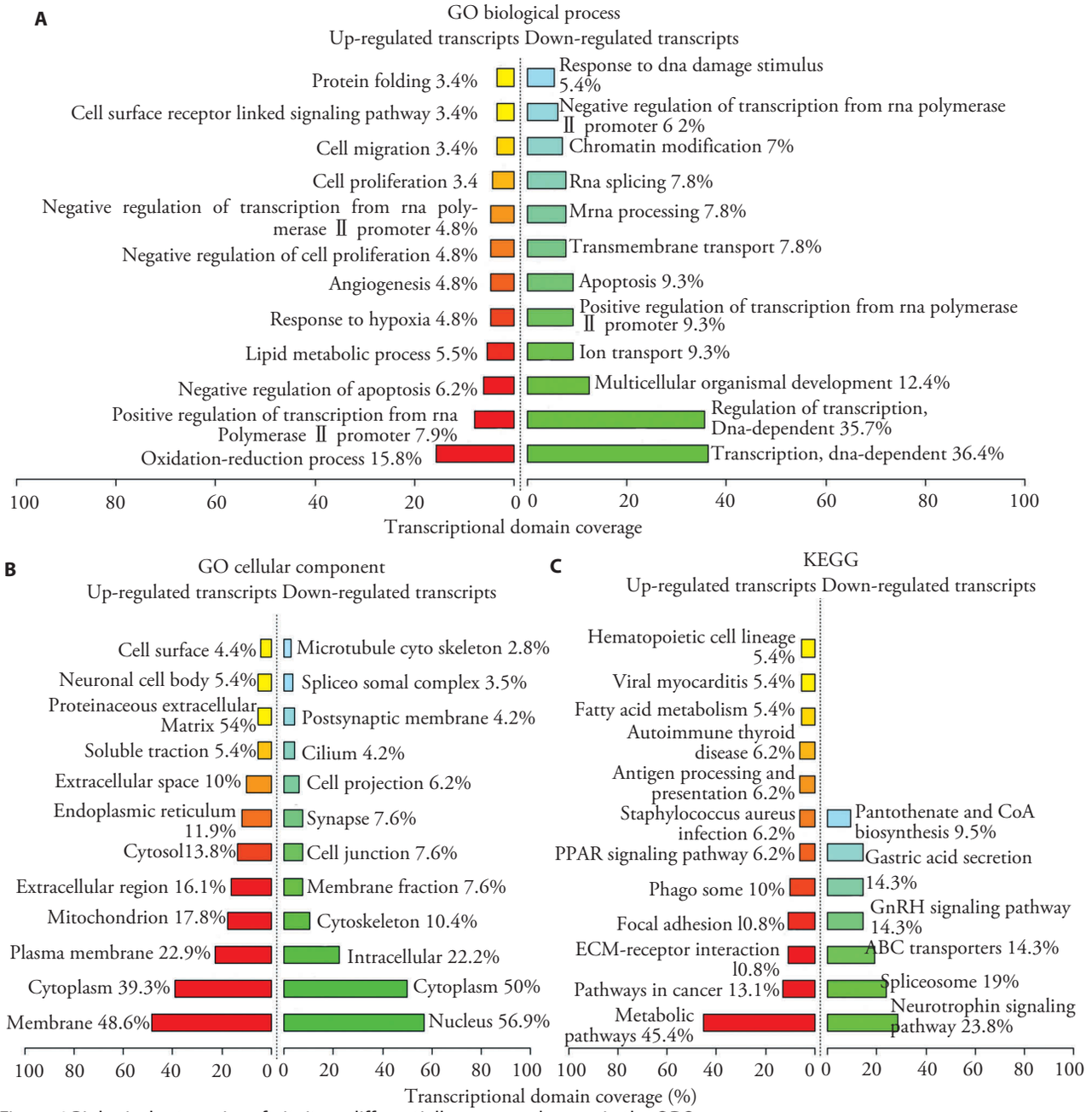

Figure 4 Biological categories of pituitary differentially expressed genes in the QDS group

A: GO biological process annotation categories of the differentially expressed genes in the QDS group; B: GO cellular component annotation categories of the differentially expressed genes in the QDS group; C: KEGG annotation categories of the differentially expressed genes in the QDS group. The percentage scale of enriched categories is presented by graded color (the categories of up-regulated genes from red to yellow versus the categories of down-regulated genes from green to blue). GO: gene ontology; KEGG: kyoto encyclopedia of genes and genomes; PPAR: peroxisome proliferator-activated receptor; GnRH: gonadotropin-releasing hormone; ABC: ATP-binding cassette; ECM: extracellular matrix; QDS: *Qi* deficiency syndrome group.

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